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Di(2-propylheptyl) phthalate – Determination of mono(2-propyl-6-carboxyhexyl) phthalate, mono(2-propyl-6-hydroxyheptyl) phthalate, and mono(2-propyl-6-oxoheptyl) phthalate in urine by GC-HR-MS or GC-MS/MS

Biomonitoring Method – Translation of the German version from 2021

Keywords

di(2-propylheptyl) phthalate; DPHP; urine; GC-HR-MS; GC-MS/MS

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Citation Note:
Gries W, Schmidtkunz C, Leng G, Müller M, Lange M, Göen T, Hartwig A, MAK Commission. Di(2-propylheptyl) phthalate – Determination of mono(2-propyl-6-carboxyhexyl) phthalate, mono(2-propyl-6-hydroxyheptyl) phthalate, and mono(2-propyl-6-oxoheptyl) phthalate in urine by GC-HR-MS or GC-MS/MS. Biomonitoring Method – Translation of the German version from 2021. MAK Collect Occup Health Saf. 2021 Sep;6(3):Doc074.
DOI: https://doi.org/10.34865/bi53306e6_3or

Manuscript completed:
18 May 2017

Publication date:
30 Sep 2021

Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method.

This method was developed for the selective detection of the main metabolites of di(2-propylheptyl) phthalate (DPHP) in urine. After enzymatic hydrolysis, the metabolites mono(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP), mono(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP), and mono(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP) are extracted from 1 ml of urine with *tert*-butyl methyl ether. The metabolites are then selectively derivatised at the carboxyl group by adding 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and diisopropylcarbodiimide. The HFIP esters formed are separated using capillary gas chromatography and analysed with subsequent high-resolution mass spectrometry or tandem mass spectrometry detection. Detection limits ranged from 0.05 to 0.1 µg/l with corresponding quantitation limits between 0.15 and 0.3 µg/l.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Gas chromatography coupled with high-resolution mass spectrometry or tandem mass spectrometry (GC-HR-MS or GC-MS/MS)

Parameters and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
Di(2-propylheptyl) phthalate (DPHP)	53306-54-0	Mono(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP)	1412411-10-9
		Mono(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP)	1372605-11-2
		Mono(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP)	1373125-92-8

Reliability data

Mono(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP) by GC-HR-MS

Within-day precision:	Standard deviation (rel.)	s_w = 1.0%, 0.8%, or 2.5%
	Prognostic range	u = 2.3%, 1.8%, or 5.5%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of cx-MPHxP per litre of urine and n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 2.2%, 4.5%, or 5.2%
	Prognostic range	u = 4.9%, 10.0%, or 11.7%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of cx-MPHxP per litre of urine and n = 10 determinations	
Accuracy:	Recovery rate (rel.)	r = 102%, 102%, or 105%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of cx-MPHxP per litre of urine and n = 10 determinations	
Detection limit:	0.05 µg cx-MPHxP per litre of urine	
Quantitation limit:	0.15 µg cx-MPHxP per litre of urine	

Mono(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP) by GC-MS/MS

Within-day precision:	Standard deviation (rel.)	s_w = 1.8%, 1.7%, or 1.1%
	Prognostic range	u = 4.6%, 4.4%, or 2.8%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of cx-MPHxP per litre of urine and n = 6 determinations	
Day-to-day precision:[a)]	Standard deviation (rel.)	s_w = 4.4%, 4.9%, or 5.6%
	Prognostic range	u = 10.4%, 11.6%, or 13.2%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of cx-MPHxP per litre of urine and n = 8 determinations	

Accuracy:	Recovery rate (rel.) at a spiked concentration of 1 µg, 10 µg, or 100 µg of cx-MPHxP per litre of urine and n = 6 determinations	r = 103%, 104%, or 106%
Detection limit:[b)]	0.05 µg cx-MPHxP per litre of urine	
Quantitation limit:[b)]	0.15 µg cx-MPHxP per litre of urine	

[a)] Day-to-day precision data were determined during external verification
[b)] See Section 11.4 and Section 11.5

Mono(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP) by GC-HR-MS

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 1 µg, 10 µg, or 100 µg of OH-MPHP per litre of urine and n = 10 determinations	s_w = 3.8%, 2.4%, or 2.9% u = 8.5%, 5.3%, or 6.4%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 1 µg, 10 µg, or 100 µg of OH-MPHP per litre of urine and n = 10 determinations	s_w = 7.4%, 4.8%, or 1.8% u = 16.4%, 10.7%, or 4.1%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 1 µg, 10 µg, or 100 µg of OH-MPHP per litre of urine and n = 10 determinations	r = 88%, 90%, or 108%
Detection limit:	0.1 µg OH-MPHP per litre of urine	
Quantitation limit:	0.3 µg OH-MPHP per litre of urine	

Mono(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP) by GC-MS/MS

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 1 µg, 10 µg, or 100 µg of OH-MPHP per litre of urine and n = 6 determinations	s_w = 6.6%, 1.4%, or 1.0% u = 17.0%, 3.6%, or 2.6%
Day-to-day precision:[a)]	Standard deviation (rel.) Prognostic range at a spiked concentration of 1 µg, 10 µg, or 100 µg of OH-MPHP per litre of urine and n = 8 determinations	s_w = 17.4%, 16.1%, or 10.7% u = 41.2%, 38.1%, or 25.3%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 1 µg, 10 µg, or 100 µg of OH-MPHP per litre of urine and n = 6 determinations	r = 115%, 106%, or 103%
Detection limit:[b)]	0.1 µg OH-MPHP per litre of urine	
Quantitation limit:[b)]	0.3 µg OH-MPHP per litre of urine	

[a)] Day-to-day precision data were determined during external verification
[b)] See Section 11.4 and Section 11.5

Mono(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP) by GC-HR-MS

Within-day precision:	Standard deviation (rel.)	s_w = 1.7%, 1.2%, or 2.0%
	Prognostic range	u = 3.9%, 2.7%, or 4.4%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of oxo-MPHP per litre of urine and n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 2.6%, 1.3%, or 1.4%
	Prognostic range	u = 5.7%, 2.8%, or 3.0%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of oxo-MPHP per litre of urine and n = 10 determinations	
Accuracy:	Recovery rate (rel.)	r = 100%, 100%, or 106%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of oxo-MPHP per litre of urine and n = 10 determinations	
Detection limit:	0.08 µg oxo-MPHP per litre of urine	
Quantitation limit:	0.25 µg oxo-MPHP per litre of urine	

Mono(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP) by GC-MS/MS

Within-day precision:	Standard deviation (rel.)	s_w = 4.7%, 1.9%, or 1.1%
	Prognostic range	u = 12.1%, 4.9%, or 2.8%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of oxo-MPHP per litre of urine and n = 6 determinations	
Day-to-day precision:[a)]	Standard deviation (rel.)	s_w = 11.0%, 10.1%, or 8.3%
	Prognostic range	u = 26.0%, 23.9%, or 19.6%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of oxo-MPHP per litre of urine and n = 8 determinations	
Accuracy:	Recovery rate (rel.)	r = 96%, 105%, or 102%
	at a spiked concentration of 1 µg, 10 µg, or 100 µg of oxo-MPHP per litre of urine and n = 6 determinations	
Detection limit:[b)]	0.1 µg oxo-MPHP per litre of urine	
Quantitation limit:[b)]	0.3 µg oxo-MPHP per litre of urine	

[a)] Day-to-day precision data were determined during external verification
[b)] See Section 11.4 and Section 11.5

2 General information on di(2-propylheptyl) phthalate (DPHP)

DPHP has a molar mass of 446.7 g/mol. At room temperature, it is a clear, colourless, and viscous liquid. The structural formula of DPHP is shown in Figure 1. DPHP, which is marketed under the trade name "Palatinol 10-P", is used as a plasticiser in PVC and vinyl chloride copolymers at concentrations of 30–60%. DPHP is used, for example, in building materials, roof coverings, cable sheathings, automotive interiors, and paints, as well as polyurethane and epoxy adhesives. Between 100 000 and 1 000 000 tons of DPHP are manufactured in or imported into the European Economic Area each year (ECHA 2021).

DPHP is produced by dimerisation of *n*-valeraldehyde, followed by hydrogenation. The alcohols formed (90% 2-propylheptanol and 10% 2-propyl-4-methylhexanol and 2-propyl-5-methylhexanol) are then esterified with phthalic anhydride. The resulting DPHP has a phthalic ester concentration of at least 99.5% with an isomer distribution of approximately 81% DPHP, 18% 2-propylheptyl-2-propyl-4/5-methylhexyl phthalate, and 1% bis(2-propyl-4/5-methylhexyl) phthalate (BfR 2011).

No studies are available on the carcinogenic effects of DPHP. Since it is suspected that long-term exposure to DPHP may cause cancer, the MAK Commission has classified DPHP in Carcinogen Category 3 (Hartwig and MAK Commission 2017). In addition, DPHP is being investigated under REACH for its possible effect as an endocrine disruptor (ECHA 2021).

DPHP is metabolised primarily via the monoester, which is oxidised by ω- and ω–1-oxidation to the hydroxy-, oxo-, and carboxymonoesters. Accordingly, 48 hours following oral administration of DPHP to volunteers, 13.5–17% of the dose was excreted with the urine as oxo-MPHP, 10.7–16% as OH-MPHP, and 0.5–3% as cx-MPHxP (Leng et al. 2014; Wittassek and Angerer 2008). The structures of the DPHP metabolites are shown in Figure 1.

The Human Biomonitoring Commission (*HBM-Kommission*) of the German Environment Agency (*Umweltbundesamt*) derived HBM values for DPHP in 2015. An HBM-I value—representing the concentration below which, according to the HBM Commission, there is no risk for adverse health effects—of 1000 µg/l for children and 1500 µg/l for adults (both in morning urine) was derived for the sum of oxo-MPHP and OH-MPHP (HBM-Kommission 2015).

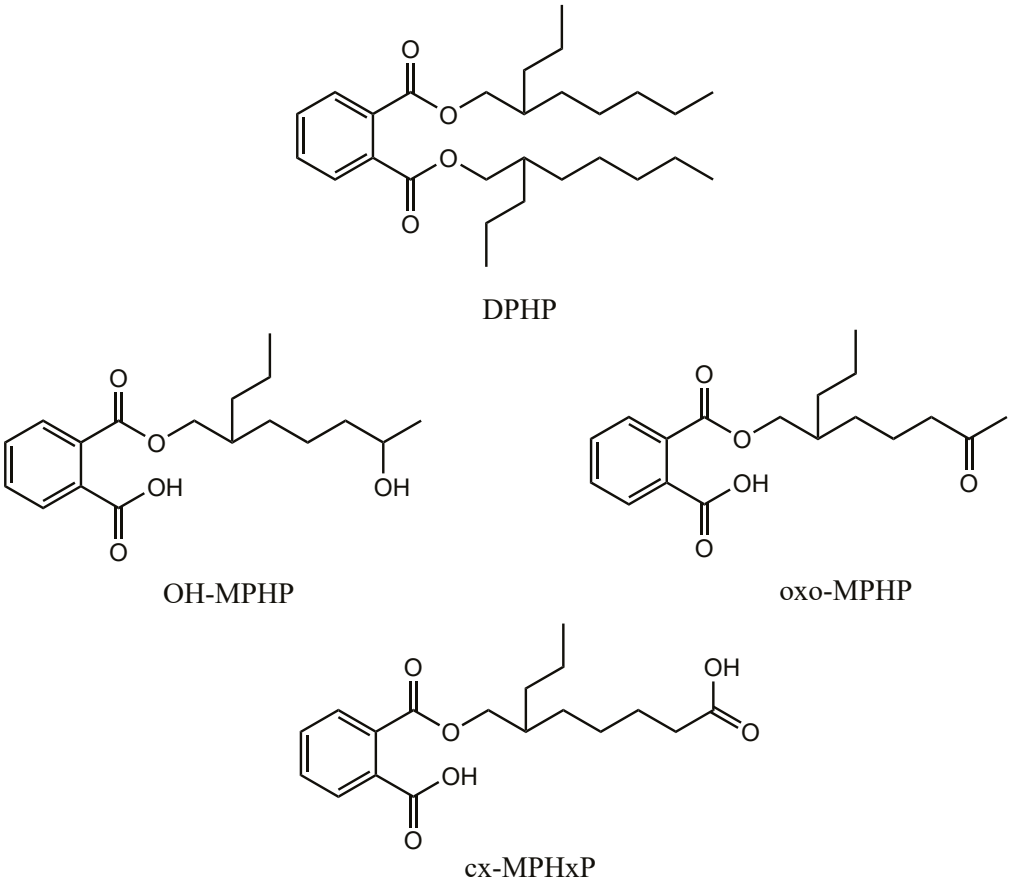

Fig. 1 Structural formulas of di(2-propylheptyl) phthalate (DPHP) and its main metabolites (per Wittassek and Angerer 2008)

With regard to the background exposure of the general population in Germany, since 2011, DPHP metabolites could be detected in about 15–22% of the urine samples in the low µg/l-range (Schmidtkunz et al. 2019). The exposure in children and adolescents from Germany is significantly higher; for instance, oxo-MPHP was detected in 62% of the samples in a GerES V study (German Environmental Survey, 2014–2017) (Schwedler et al. 2020 a). A research group in Finland investigated OH-MPHP concentrations in the urine of workers after occupational exposure to DPHP (Porras et al. 2020).

The OH-MPHP concentrations, determined in the post-shift urine samples of the workers, are given in Table 1, along with the data from the general population studies mentioned above.

Tab. 1 Concentrations of cx-MPHxP, OH-MPHP, and oxo-MPHP in urine of the general population and after occupational exposure to DPHP

Group (number of samples, n)	cx-MPHxP	OH-MPHP	oxo-MPHP	References
	Median (range) [µg/l]	Median (range) [µg/l]	Median (range) [µg/l]	
General population, Germany 2011 (40)	< LOQ (< LOQ–< LOQ)	< LOQ (< LOQ–0.51); 8% of samples > LOQ	< LOQ (< LOQ–0.93); 38% of samples > LOQ	Gries et al. 2012
Young adults, Germany 2009 (60)	< LOQ (< LOQ–< LOQ)	< LOQ (< LOQ–0.64); 3.3% of samples > LOQ	< LOQ (< LOQ–0.96); 3.3% of samples > LOQ	Schütze et al. 2015
Young adults, Germany 2012 (60)	< LOQ (< LOQ–< LOQ)	< LOQ (< LOQ–0.36); 3.3% of samples > LOQ	< LOQ (< LOQ–0.65); 21.7% of samples > LOQ	
Young adults, Germany 2011 (60)	< LOQ (< LOQ–< LOQ)	< LOQ (< LOQ–0.45); 1.7% of samples > LOQ	< LOQ (< LOQ–0.69); 18.3% of samples > LOQ	Schmidtkunz et al. 2019
Young adults, Germany 2014 (60)	< LOQ (< LOQ–< LOQ)	< LOQ (< LOQ–0.46); 3.3% of samples > LOQ	< LOQ (< LOQ–0.81); 15.0% of samples > LOQ	
Young adults, Germany 2017 (60)	< LOQ (< LOQ–< LOQ)	< LOQ (< LOQ–0.32); 1.7% of samples > LOQ	< LOQ (< LOQ–1.45); 18.3% of samples > LOQ	
Children and adolescents, Germany 2014–2017 (516)	< LOQ (< LOQ–0.83); 0.8% of samples > LOQ	< LOQ (< LOQ–17.9); 50% of samples > LOQ	0.31 (< LOQ–27.6); 62% of samples > LOQ	Schwedler et al. 2020 a
Cable factory (5)	n. a.	3.9 (1.8–7.8)[a)]	n. a.	Porras et al. 2020
Plastics production (5)	n. a.	7.1 (< LOQ–21.0)[a)]	n. a.	

LOQ: limit of quantitation; n. a.: not analysed

[a)] As OH-MPHP concentration levels were quantified by LC-MS/MS, hydroxy-metabolites of DPHP were not differentiated from those of further C10-phthalates

3 General principles

This method was developed for the selective detection of the main metabolites of DPHP in urine. After enzymatic hydrolysis, the released metabolites cx-MPHxP, OH-MPHP, and oxo-MPHP are extracted from 1 ml of urine with *tert*-butyl methyl ether. The metabolites are then selectively derivatised at the carboxyl group by adding HFIP and diisopropylcarbodiimide. The HFIP esters thus formed are separated using capillary gas chromatography and analysed with subsequent HR-MS or MS/MS detection. Detection limits ranged from 0.05 to 0.1 µg/l with corresponding quantitation limits between 0.15 and 0.3 µg/l. Figure 2 shows the reaction mechanism of the esterification of the carboxylic acid with HFIP.

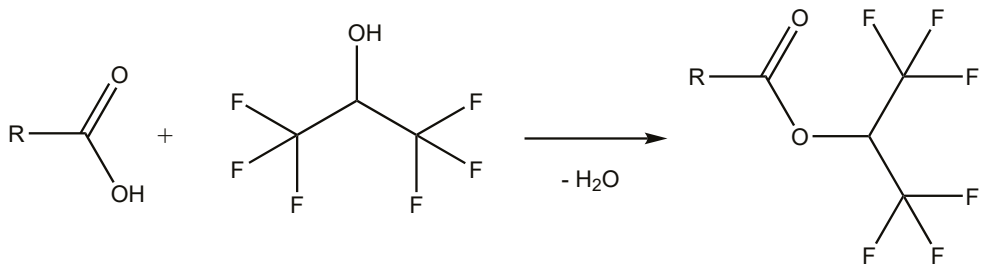

Fig. 2 Schematic representation of the esterification reaction of the carboxylic acid with HFIP

4 Equipment, chemicals, and solutions

4.1 Equipment

- Gas chromatograph (e.g. Agilent GC 5890, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) with an autosampler (e.g. CTC A200S, CTC Analytics AG, Zwingen, Switzerland), and an HR-MS detector (e.g. Waters AutoSpec Ultima mass spectrometer, Waters GmbH, Eschborn, Germany)
- Gas chromatograph (e.g. CP-3800, Varian Inc., Palo Alto, USA) with MS/MS detector (e.g. 300 MS-TQ, Varian Inc., Palo Alto, USA)
- GC column: Rxi®-17, 30 m × 0.25 mm × 0.25 µm or Rtx-65, 30 m × 0.25 mm × 0.25 µm (e.g. Restek GmbH, Bad Homburg v. d. Höhe, Germany)
- 200-µl vials with crimp caps (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- Analytical balance (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Incubator (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- 3.5-ml disposable pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Refrigerated centrifuge (e.g. Thermo Fisher Scientific GmbH, Dreieich, Germany)
- 10-ml and 500-ml volumetric flasks (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 250-ml and 400-ml beakers (e.g. VWR International GmbH, Darmstadt, Germany)
- Multipette® (e.g. Eppendorf AG, Hamburg, Germany)
- Polyethylene Pasteur pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- pH meter (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- 12-ml sample vials with Teflon-coated screw caps (e.g. VWR International GmbH, Darmstadt, Germany)
- Laboratory shaker (e.g. Vibrax VXR, IKA®-Werke GmbH & Co. KG, Staufen, Germany)
- Nitrogen evaporator (e.g. Zymark, Biotage AB, Uppsala, Sweden)
- 10-µl, 100-µl, and 1000-µl transfer pipettes (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- Urine-collection cups (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

- 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) (e.g. No. 845157, Merck KGaA, Darmstadt, Germany)
- Acetonitrile SupraSolv® (e.g. No. 100017, Merck KGaA, Darmstadt, Germany)
- Ammonium acetate (e.g. No. 32301-M, Merck KGaA, Darmstadt, Germany)
- *β*-Glucuronidase *E. coli* K12 in 50% glycerol, ≥ 80 U/mg protein (e.g. No. 3707598001, Roche Diagnostics Deutschland GmbH, Mannheim, Germany)
- Glacial acetic acid (e.g. No. 1.00063.1011, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q® plus VE system (> 18 MΩ), Merck KGaA, Darmstadt, Germany)
- Isooctane for residue analysis (e.g. No. 650439, Merck KGaA, Darmstadt, Germany)

- Mono(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP) > 95% (e.g. No. 2071, IDM, Teltow, Germany)
- Mono(2-propyl-6-carboxyhexyl) phthalate, quadruple ring-deuterated (cx-MPHxP-d_4) > 95% (e.g. No. 2072, IDM, Teltow, Germany)
- Mono(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP) > 95% (e.g. No. 2067, IDM, Teltow, Germany)
- Mono(2-propyl-6-hydroxyheptyl) phthalate, quadruple ring-deuterated (OH-MPHP-d_4) > 95% (e.g. No. 2068, IDM, Teltow, Germany)
- Mono(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP) > 90% (e.g. No. 2069, IDM, Teltow, Germany)
- Mono(2-propyl-6-oxoheptyl) phthalate, quadruple ring-deuterated (oxo-MPHP-d_4) > 90% (e.g. No. 2070, IDM, Teltow, Germany)
- Sodium hydrogen carbonate (e.g. No. 1.06329, Merck KGaA, Darmstadt, Germany)
- *N,N´*-Diisopropylcarbodiimide > 98% (e.g. No. 38370, Merck KGaA, Darmstadt, Germany)
- Hydrochloric acid 37% (e.g. No. 1.00317, Merck KGaA, Darmstadt, Germany)
- *tert*-Butyl methyl ether for residue analysis (e.g. No. 1.01849, Merck KGaA, Darmstadt, Germany)

4.3 Solutions

- Sodium hydrogen carbonate solution (1 mol/l)

  42 g of sodium hydrogen carbonate are weighed into a 250-ml beaker, partly dissolved in approximately 100 ml of ultra-pure water, and quantitatively transferred into a 500-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.

- Ammonium acetate solution (1 mol/l, pH 6.5)

  38.5 g of ammonium acetate are weighed into a 400-ml beaker and dissolved in approximately 250 ml of ultra-pure water. Using a pH meter, the pH is adjusted to pH 6.5 with glacial acetic acid. The solution is quantitatively transferred into a 500-ml volumetric flask, which is then made up to the mark with ultra-pure water.

When stored in the refrigerator at 4 °C, the solutions are stable for at least three months.

4.4 Internal standards (ISTDs)

- ISTD stock solutions (1000 mg/l)

  10 mg each of cx-MPHxP-d_4, OH-MPHP-d_4, and oxo-MPHP-d_4 are weighed exactly into separate 10-ml volumetric flasks and dissolved in acetonitrile. The volumetric flasks are then made up to the mark with acetonitrile.

- ISTD working solution (10 mg/l)

  100 µl of each ISTD stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

- ISTD spiking solution (1 mg/l)

  1 ml of the ISTD working solution is pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

When stored in the refrigerator at 4 °C, the solutions of the internal standards are stable for at least twelve months.

4.5 Calibration standards

- Stock solutions (1000 mg/l)

  10 mg each of cx-MPHxP, OH-MPHP, and oxo-MPHP are weighed exactly into separate 10-ml volumetric flasks and dissolved in acetonitrile. The volumetric flasks are then made up to the mark with acetonitrile.

- Spiking solution I (10 mg/l)

  100 µl of each stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

- Spiking solution II (1 mg/l)

  1 ml of spiking solution I is pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

- Spiking solution III (0.1 mg/l)

  100 µl of spiking solution I are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

- Spiking solution IV (0.01 mg/l)

  100 µl of spiking solution II are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

- Spiking solution V (0.001 mg/l)

  1 ml of spiking solution IV is pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

When stored in the refrigerator at 4 °C, the stock and spiking solutions of the analytes are stable for at least twelve months.

Calibration standards, in the concentration range of 0.05 to 200 µg/l, are prepared in pooled urine from persons without known exposure to DPHP based on the pipetting scheme given in Table 2. Unspiked pooled urine is included as a blank value. Calibration up to 50 µg/l is usually sufficient for method application in the field of environmental medicine.

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of DPHP metabolites in urine

Calibration standard	Spiking solution	Volume of spiking solution [µl]	Volume of pooled urine [µl]	Analyte concentration [µg/l]
0	–	0	1000	0
1	V	50	950	0.05
2	IV	10	990	0.1
3	IV	20	980	0.2
4	IV	50	950	0.5
5	III	10	990	1
6	III	20	980	2
7	III	50	950	5
8	II	10	990	10
9	II	20	980	20

Tab. 2 *(continued)*

Calibration standard	Spiking solution	Volume of spiking solution [µl]	Volume of pooled urine [µl]	Analyte concentration [µg/l]
10	II	50	950	50
11	I	10	990	100
12	I	20	980	200

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in sealable plastic containers and frozen at –20 °C until analysis. Prior to analysis, the samples are brought to room temperature and thoroughly mixed.

5.2 Sample preparation

1 ml of each urine sample is pipetted into a 12-ml sample vial. After adding 10 µl of the ISTD spiking solution, 2 ml of ammonium acetate solution, and 10 µl of *β*-glucuronidase, the samples are incubated overnight at 37 °C in an incubator. After the samples have been cooled to room temperature, they are acidified (pH < 2) by adding 200 µl of concentrated hydrochloric acid. For extraction, 4 ml of *tert*-butyl methyl ether are added and the samples are shaken on a shaking table for 10 minutes. For improved phase separation, the samples are centrifuged at 2200 × *g* for 10 minutes in a refrigerated centrifuge (10 °C). The *tert*-butyl methyl ether phase is then transferred to a new sample vial using a Pasteur pipette and evaporated to dryness under a stream of nitrogen at 35 °C.

The residue is redissolved in 150 µl of acetonitrile. After adding of 20 µl of HFIP and 10 µl of diisopropylcarbodiimide, the sample vials are sealed and the samples are derivatised for 15 min at room temperature. Afterwards, 1 ml of the sodium hydrogen carbonate solution and 500 µl of isooctane are added and the samples are vigorously shaken for 10 min. To facilitate phase separation, the samples are centrifuged at 2200 × *g* for 10 minutes in a refrigerated centrifuge (10 °C). The isooctane phase is then transferred to a 200-µl vial and applied for analysis by GC-HR-MS or GC-MS/MS.

6 Operational parameters

Analysis is performed using a device configuration comprised of a gas chromatograph coupled with a high-resolution mass spectrometer (GC-HR-MS) or with a tandem mass spectrometer (GC-MS/MS).

6.1 Gas chromatography

Capillary column:	Stationary phase:	Rxi®-17 (diphenyl-/dimethylpolysiloxane (50%/50%)) alternatively: Rtx-65 (diphenyl-/dimethylpolysiloxane (65%/35%))
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm

Temperature:	Column:	Initial temperature of 90 °C, hold for 1 min, increase at a rate of 12 °C/min to 250 °C, then increase at a rate of 30 °C/min to 280 °C, hold for 5 min
	Injector:	280 °C
Carrier gas:	Helium 5.0	
Pressure:	120 kPa	
Injection:	1 µl, splitless, 1 min	
Insert:	Double Gooseneck	4 mm inner diameter

6.2 Mass spectrometry

High-resolution mass spectrometry

Ionisation type:	Negative chemical ionisation (NCI)
NCI gas:	Ammonia gas
CI gas flow:	2×10^{-5} mbar
Transfer line:	250 °C
Ion-source temperature:	230 °C
Emission current:	0.5 mA
Resolution:	5000
Multiplier:	350 V
Electron energy:	100 eV
Parameter-specific settings:	see Table 3

Tab. 3 Parameter-specific settings and retention times of the analytes and internal standards for the determination of DPHP metabolites in urine by GC-HR-MS

Analyte / ISTD	Retention time[a] [min]	Mass trace [*m/z*]
cx-MPHxP	11.51	485.1399
cx-MPHxP-d_4	11.50	489.1650
OH-MPHP	14.01	321.1762
OH-MPHP-d_4	14.00	325.1953
oxo-MPHP	14.09	319.1545
oxo-MPHP-d_4	14.08	323.1797

[a] Rtx-65 separation column

Figure 3 shows representative HR mass spectra of the HFIP derivatives of cx-MPHxP, OH-MPHP, and oxo-MPHP. The ions used for quantification arise from the cleavage of a $CH(CF_3)_2$ moiety (*m/z* 150.9982).

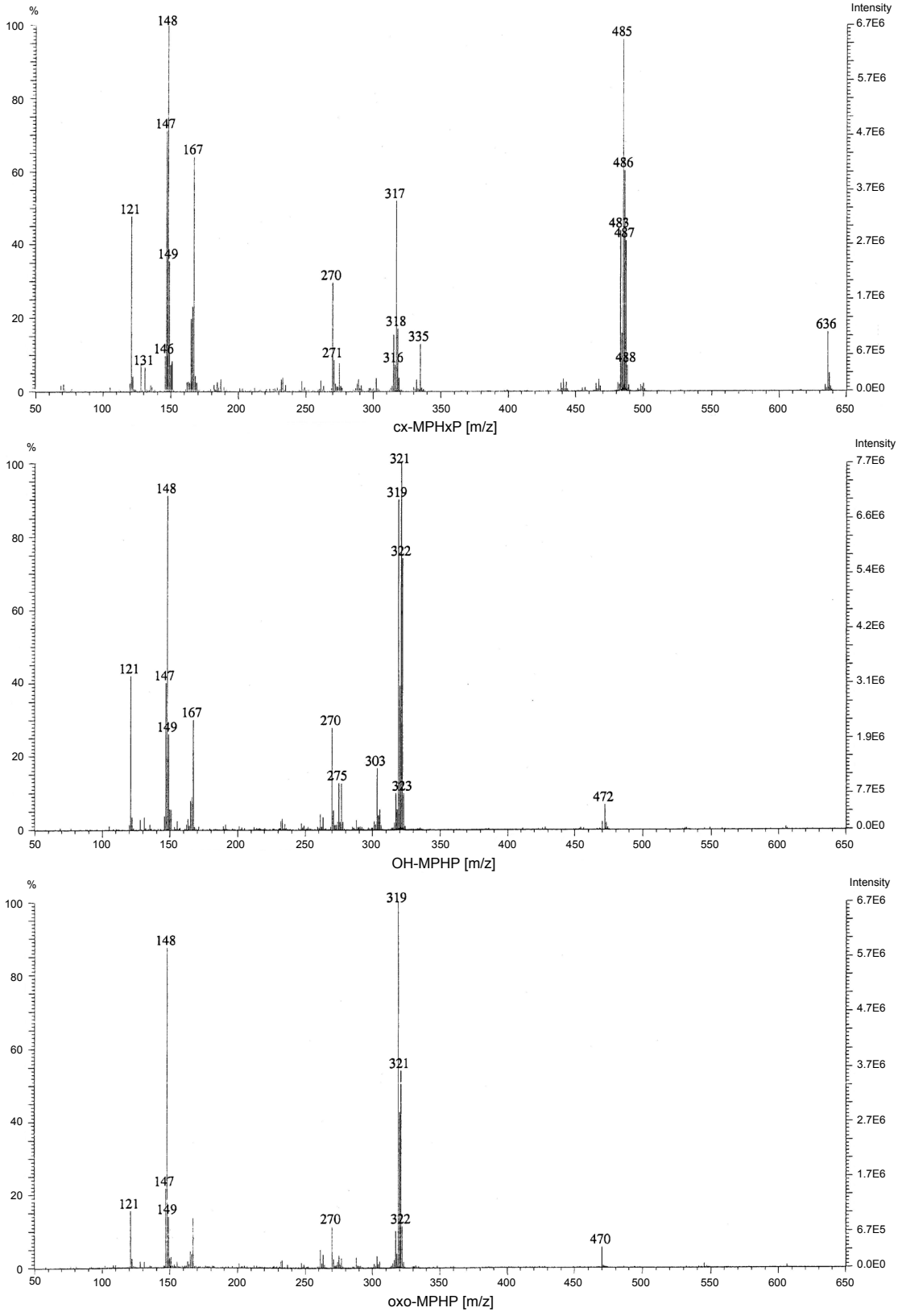

Fig. 3 HR mass spectra of the HFIP derivatives of cx-MPHxP, OH-MPHP, and oxo-MPHP

Triple-quadrupole mass spectrometry

Ionisation type:	Negative chemical ionisation (NCI)
NCI gas:	Methane
CI gas flow:	4.7 mbar
Transfer line:	280 °C
Ion-source temperature:	250 °C
CID gas:	Argon
CID gas flow:	2.4×10^{-6} bar
Multiplier:	1200 V
Electron energy:	100 eV
Parameter-specific settings:	see Table 4

Tab. 4 Parameter-specific settings and retention times of the analytes and internal standards for the determination of DPHP metabolites in urine by GC-MS/MS

Analyte / ISTD	Retention time[a)] [min]	Precursor ion Q1 [*m/z*]	Product ion Q3 [*m/z*]	Collision energy [V]
cx-MPHxP	12.15	485	317	15
cx-MPHxP-d_4	12.14	489	321	15
OH-MPHP	14.09	321	121	20
OH-MPHP-d_4	14.07	325	125	20
oxo-MPHP	14.22	319	121	20
oxo-MPHP-d_4	14.20	323	125	20

[a)] Rxi®-17 separation column

7 Analytical determination

1 µl of each sample prepared as described in Section 5 is injected into the GC-HR-MS or GC-MS/MS system. Identification of the analytes is based on their specific ions or ion transitions and retention times (see Tables 3 and 4). The retention times given in Tables 3 and 4 are intended only as cursory guidance. Users must ensure proper separation performance of the column used as well as the resulting retention behaviour of the analytes. Figures 4 and 5 show representative chromatograms of a native urine sample and a urine sample spiked with 1 µg/l of the respective DPHP metabolites (GC-MS/MS).

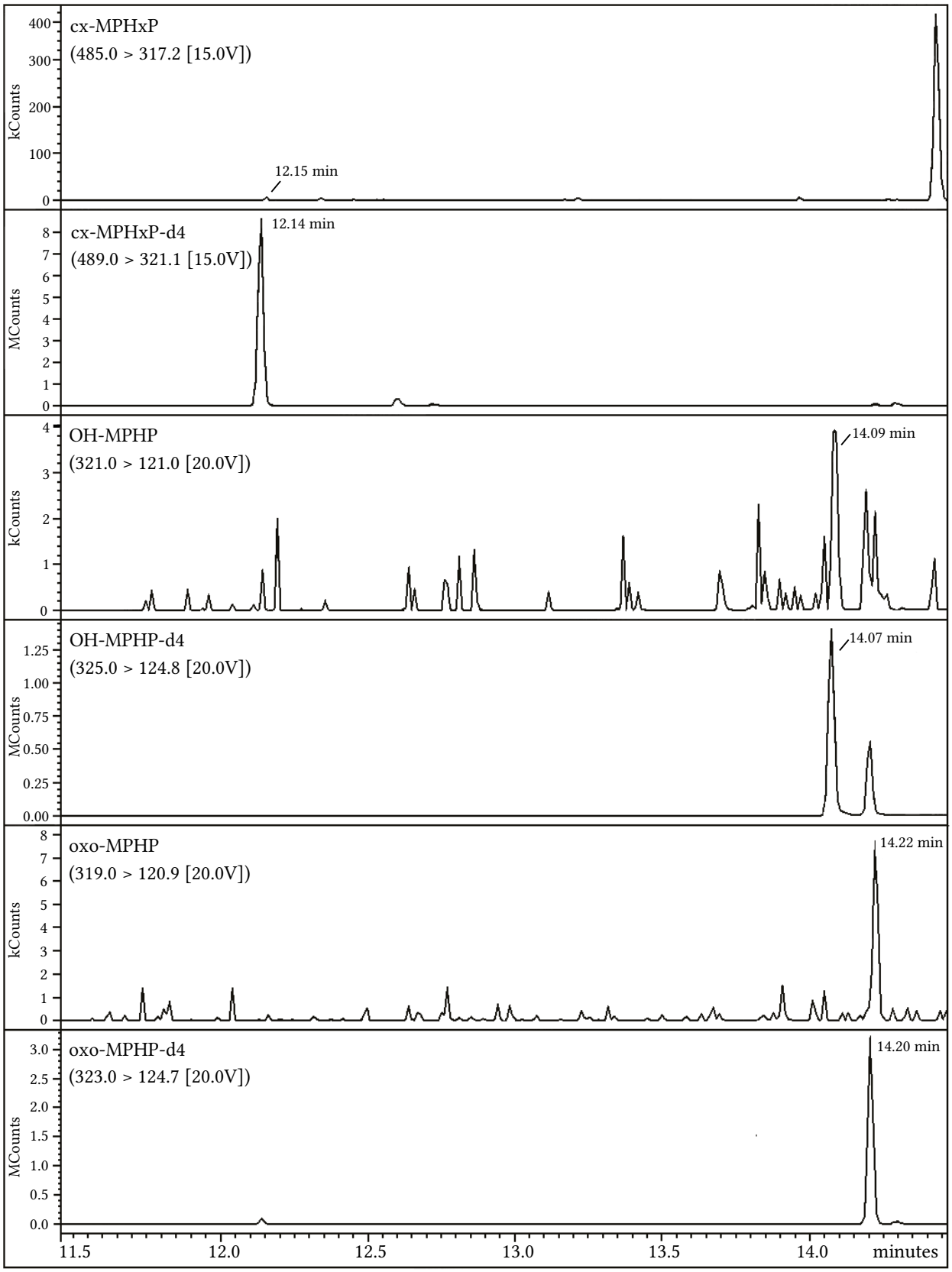

Fig. 4 Chromatogram of a native urine sample (GC-MS/MS)

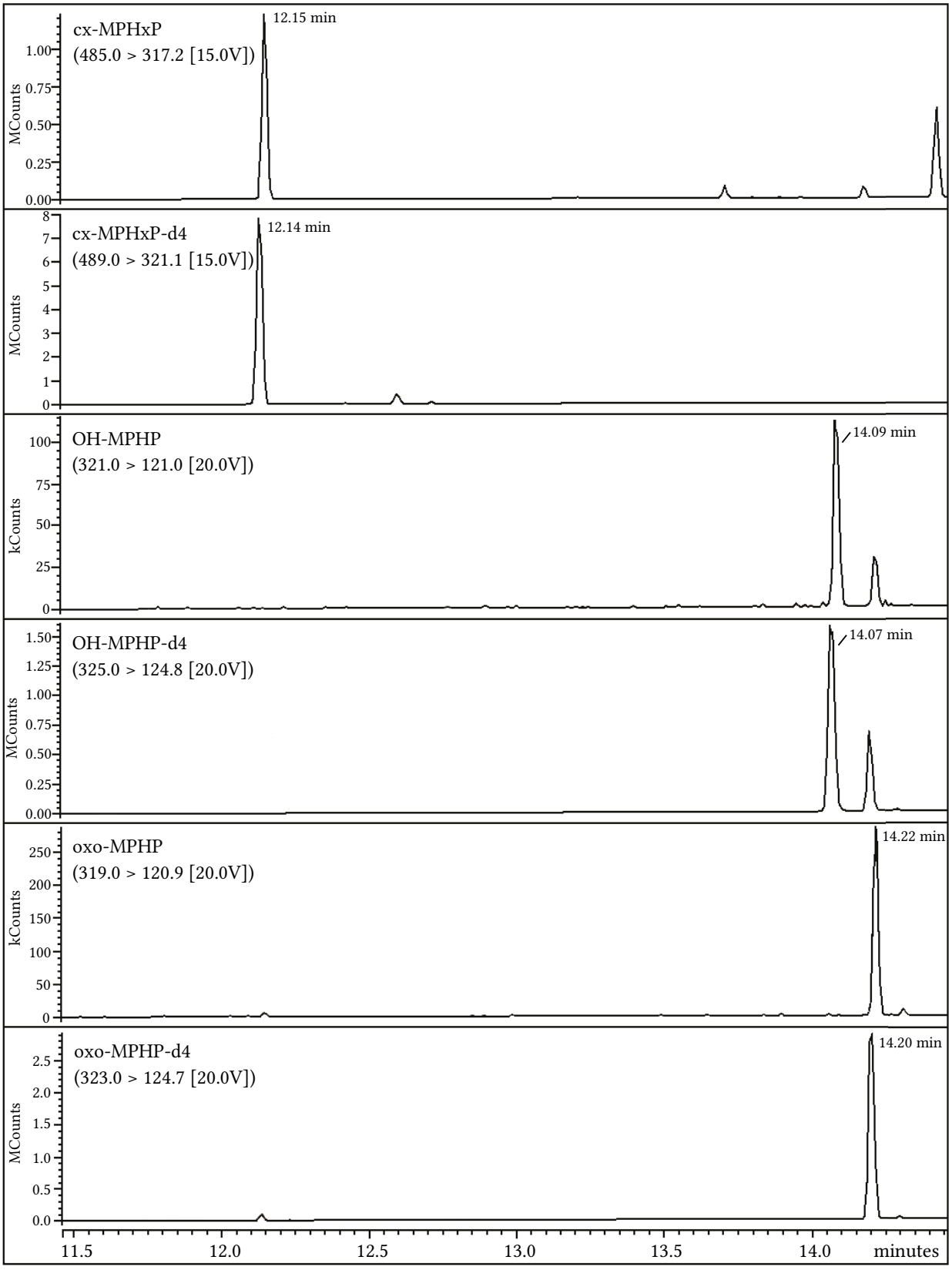

Fig. 5 Chromatogram of a urine sample spiked with 1 µg/l of the respective DPHP metabolites (GC-MS/MS)

8 Calibration

For calibration of the method, the calibration standards prepared as described in Section 4.5 are processed analogously to the samples (cf. Section 5) and analysed (cf. Sections 6 and 7). Calibration curves are constructed by plotting the quotient of the peak area of the respective DPHP metabolite and the corresponding internal standard against the spiked concentration of the respective calibration standard. For HR-MS detection, the linear range of measurement lied between the detection limit and 20 µg/l for cx-MPHxP or 50 µg/l for OH-MPHP and oxo-MPHP. For MS/MS detection, a linear range of measurement from the detection limit to 200 µg/l was determined for all DPHP metabolites.

Linear regression is used to calculate the function of the calibration curve. Any blank values from the sample material must be subtracted from all points of measurement. Figure 6 shows representative calibration curves for the determination of DPHP metabolites in urine (GC-MS/MS).

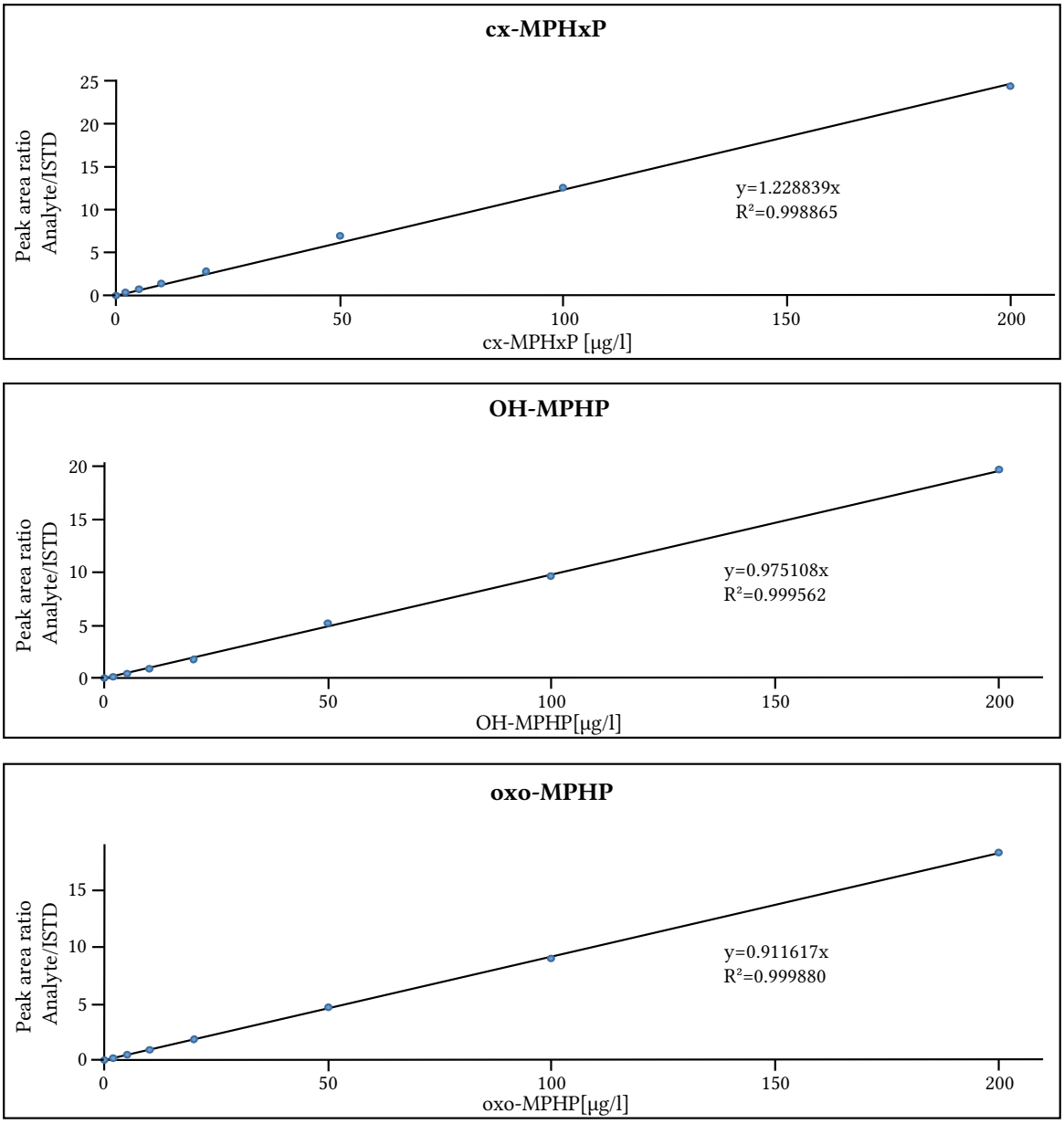

Fig. 6 Calibration curves for the determination of DPHP metabolites in urine by GC-MS/MS

9 Calculation of the analytical results

The analyte concentration in a urine sample is calculated by dividing the peak area of the analyte by the peak area of the ISTD. Using the calibration function of the corresponding analytical run (Section 8), the analyte concentration in µg/l of urine can be calculated with the quotient thus obtained. If the analytical result lies above the calibration range, the sample is diluted with ultra-pure water, reprocessed, and newly analysed.

A reagent blank (ultra-pure water) is included in each analytical run. Any reagent blank values must be subtracted from the analytical results.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

To check precision, at least two quality-control samples with known analyte concentrations are included in each analytical run. As quality-control material is not commercially available, it must be prepared by spiking pooled urine from persons with no known exposure to DPHP with standard solutions of the analytes. The concentrations of the analytes in the quality-control materials should lie within the relevant concentration range (e.g. 1 µg/l and 10 µg/l). Aliquots of these samples are stored at –20 °C and are included in each analytical run as quality-control samples. The nominal values and the tolerance ranges of the quality-control materials are determined in a pre-analytical period (one analysis of each control material on ten different days) (Bader et al. 2010).

At the same time, ultra-pure water is additionally included in each analytical run as a reagent blank in order to detect any interferences caused by the reagents.

11 Evaluation of the method

The method was originally developed as a GC-HR-MS method and the reliability of the method was confirmed by comprehensive validation. Additional validation data were collected for MS/MS detection by the developers of the method. The GC-MS/MS method was verified by implementation and replication in a second, independent laboratory.

11.1 Precision

Within-day precision

Urine from a person not occupationally exposed to DPHP was used to determine within-day precision. 1-ml urine samples were spiked with analyte concentrations of 1 µg/l, 10 µg/l, and 100 µg/l. These samples were processed (Section 5.2) and analysed (Sections 6 and 7) as described. The within-day precision data ascertained by HR-MS or MS/MS detection are presented in Tables 5 and 6, respectively.

Tab. 5 Within-day precision for the determination of DPHP metabolites in urine by GC-HR-MS (n = 10)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MPHxP	1	1.0	2.3
	10	0.8	1.8
	100	2.5	5.5
OH-MPHP	1	3.8	8.5
	10	2.4	5.3
	100	2.9	6.4
oxo-MPHP	1	1.7	3.9
	10	1.2	2.7
	100	2.0	4.4

Tab. 6 Within-day precision for the determination of DPHP metabolites in urine by GC-MS/MS (n = 6)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MPHxP	1	1.8	4.6
	10	1.7	4.4
	100	1.1	2.8
OH-MPHP	1	6.6	17.0
	10	1.4	3.6
	100	1.0	2.6
oxo-MPHP	1	4.7	12.1
	10	1.9	4.9
	100	1.1	2.8

Day-to-day precision

For the GC-HR-MS method, urine from a person not occupationally exposed to DPHP was spiked with analyte concentrations of 1 µg/l, 10 µg/l, and 100 µg/l. On each of five different days, two urine samples were processed and analysed. The precision data thus obtained are presented in Table 7.

For the GC-MS/MS method, the day-to-day precision data was collected by the verifiers of the method. For this purpose, urine from a person not occupationally exposed to DPHP was spiked with the relevant analytes at concentrations of 1 µg/l, 10 µg/l, or 100 µg/l. These urine samples were processed and analysed on eight different days. The precision data thus obtained are given in Table 8.

Tab. 7 Day-to-day precision for the determination of DPHP metabolites in urine by GC-HR-MS (n = 10)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MPHxP	1	2.2	4.9
	10	4.5	10.0
	100	5.2	11.7
OH-MPHP	1	7.4	16.4
	10	4.8	10.7
	100	1.8	4.1
oxo-MPHP	1	2.6	5.7
	10	1.3	2.8
	100	1.4	3.0

Tab. 8 Day-to-day precision for the determination of DPHP metabolites in urine by GC-MS/MS (n = 8)[a)]

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MPHxP	1	4.4	10.4
	10	4.9	11.6
	100	5.6	13.2
OH-MPHP	1	17.4	41.2
	10	16.1	38.1
	100	10.7	25.3
oxo-MPHP	1	11.0	26.0
	10	10.1	23.9
	100	8.3	19.6

[a)] Day-to-day precision data for detection by GC-MS/MS were determined during external verification

11.2 Accuracy

The accuracy of the methods was ascertained from the within-day precision data. The relative recovery rates thus obtained are given in Table 9 for HR-MS detection and in Table 10 for MS/MS detection.

Tab. 9 Mean relative recovery for the determination of DPHP metabolites in urine by GC-HR-MS (n = 10)

Analyte	Spiked concentration [µg/l]	Mean rel. recovery r [%]	Range [%]
cx-MPHxP	1	102	100–104
	10	102	101–103
	100	105	98–107
OH-MPHP	1	88	82–92
	10	90	88–95
	100	108	95–108
oxo-MPHP	1	100	98–104
	10	100	98–102
	100	106	101–109

Tab. 10 Mean relative recovery for the determination of DPHP metabolites in urine by GC-MS/MS (n = 6)

Analyte	Spiked concentration [µg/l]	Mean rel. recovery r [%]	Range [%]
cx-MPHxP	1	103	101–106
	10	104	101–105
	100	106	105–108
OH-MPHP	1	115	104–123
	10	106	104–108
	100	103	102–105
oxo-MPHP	1	96	89–103
	10	105	101–106
	100	102	100–104

11.3 Matrix effects

Ten different urine samples from persons with no known exposure to DPHP were used to assess the robustness towards potential matrix effects. The urine samples contained creatinine in the range of 0.52 g/l to 3.74 g/l and were each spiked with 10 µg/l of the DPHP metabolites, processed as described (Section 5.2), and analysed by GC-HR-MS (Sections 6 and 7). Relative standard deviations ranged from 3.8% to 4.0% and mean relative recoveries were between 100% and 103%. Hence, the varying compositions of the urine samples had no relevant impact on the analytical results.

11.4 Limits of detection and quantitation

For HR-MS detection, the limits of detection and quantitation were determined based on the calibration-curve method following DIN 32645 (DIN 2008) using the six lowest points of the calibration curve (0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 µg/l, n = 3). The data are presented in Table 11. For MS/MS detection, the detection and quantitation limits ascertained by the developers of the method were within the same range (Table 12). In practice, several factors influence the detection and quantitation limits which can actually be achieved. Users must ensure proper detection sensitivity of the GC-HR-MS or GC-MS/MS instrument, if low limits of quantitation are relevant for the intended analyses. More information is provided in Section 11.5.

Tab. 11 Limits of detection and quantitation for the determination of DPHP metabolites in urine by GC-HR-MS (n = 3)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
cx-MPHxP	0.05	0.15
OH-MPHP	0.1	0.3
oxo-MPHP	0.08	0.25

Tab. 12 Limits of detection and quantitation for the determination of DPHP metabolites in urine by GC-MS/MS (n = 3)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
cx-MPHxP	0.05	0.15
OH-MPHP	0.1	0.3
oxo-MPHP	0.1	0.3

11.5 Sources of error

According to the manufacturer, the optimal activity range of the *β*-glucuronidase used lies between pH 6.0 and 6.5 at an incubation temperature of 37 °C. Under these conditions, the cleavage process is very rapid and is completed within 90 min at maximum. This method uses overnight hydrolysis as this was easier to establish in laboratory routines. It is not strictly necessary to carry out this step analogously.

The analytical method described herein involves a derivatisation step with HFIP. Esterification, with introduction of the $CH(CF_3)_2$ group, only proceeds quantitatively in an anhydrous medium. It is therefore important to ensure that the removed *tert*-butyl methyl ether phase is free of water.

It was found that the use of non-polar GC-columns, such as a Rtx-5 column, leads to peak tailing for OH-MPHP. OH-MPHP and oxo-MPHP cannot be completely separated using this phase. The mass fragments formed may thus overlap, which limits selective quantification. This observation led to the use of the mid-polar separation phases Rxi®-17 and Rtx-65, which allow optimal peak resolution and separation of the DPHP metabolites without additional derivatisation of the OH group of OH-MPHP. The separation performance of both phases is similar, but the use of the Rxi®-17 phase is more advantageous in the long term due to its higher temperature stability.

Background signals were observed in the reagent blanks which can be attributed to the internal standards. They lied within the orders of magnitude of 0.1 µg/l for cx-MPHxP, 0.5 µg/l for OH-MPHP, and 0.2 µg/l for oxo-MPHP. These background signals must be subtracted when generating the calibration curve as well as when calculating the metabolite concentrations in the samples to be analysed. These background signals have a disadvantageous effect on the calculation of the detection and quantification limits, as the signal increase becomes blurred in the lower range of measurement, which makes it difficult to achieve lower limits of detection and quantitation.

No significant matrix interferences were observed during method development, except for possible interfering peaks caused by urinary background levels of metabolites of diisodecyl phthalate (DiDP) or diisononyl phthalate (DiNP). Technical DiDP and DiNP are both mixtures containing substances which are isomers of DPHP (especially in the case of DiDP). Metabolism of these plasticisers may hence lead to isomers of the DPHP metabolites cx-MPHxP, OH-MPHP, and oxo-MPHP. These isomers form isobaric ions in mass spectrometry and can therefore cause interferences. The background exposure with DiDP/DiNP is significantly higher than DPHP levels, meaning that the quality of the chromatographic separation is crucial for the selective detection of DPHP exposure (Gries et al. 2012; Koch et al. 2017; Schwedler et al. 2020 b).

The GC-HR-MS or GC-MS/MS method presented herein allows for a reliable separation of cx-MPHxP and oxo-MPHP from the respective isomeric metabolites of DiDP/DiNP, whereas the selectivity for OH-MPHP is insufficient if DiDP/DiNP exposure is at a high level – this holds true even when GC-HR-MS is performed with a 60-m column (Gries et al. 2012). The application of the method in large population studies (e.g. Schmidtkunz et al. 2019) confirmed both the very robust and highly selective detection of oxo-MPHP as well as the selectivity issues hampering OH-MPHP quantification in individual urine samples with high DiDP/DiNP metabolite levels. The limit of quantitation for OH-MPHP (0.3 µg/l) cannot be guaranteed if DiDP/DiNP metabolite concentrations are high. On the other hand, cx-MPHxP is a minor metabolite, and hence it is only detectable after very significant exposure to DPHP. At trace levels common in environmental medicine, oxo-MPHP is thus the best biomarker for DPHP exposure. It combines good detection selectivity with a low limit of quantitation. Additionally, it is the major oxidized urinary DPHP metabolite, accounting for 13.5% of an oral DPHP dose (Leng et al. 2014).

During external method verification, worse precision data and recovery rates were obtained for the spiked 1 µg/l urine samples than indicated by the developers of the method. Relative standard deviations were determined to be between 4.4% and 22.6% and the relative recovery rates were in the range of 157% to 239% (n = 8). Accordingly, limits of detection and quantitation were about one order of magnitude higher than the limits reported by the developers of the method. This is probably due to the fact that an additional drying step of the isooctane extract with 0.2 g of sodium sulphate was carried out during external method verification. Furthermore, the use of a single gooseneck insert instead of a double gooseneck insert may play a role. Therefore, the use of a double gooseneck insert is recommended. Moreover, the drying step can be omitted, since it is also possible to work with an aliquot of the isooctane phase after the extraction step.

Generally, the analyte concentrations expected in the environmental range (< 1 µg/l, very rarely up to about 30 µg/l) require special attention as to the production of the extracts and, if necessary, the fine-tuning of the corresponding analytical device.

12 Discussion of the method

The analytical method presented herein enables the selective and sensitive determination of the main DPHP metabolites using a GC-HR-MS or a GC-MS/MS technique with negative chemical ionisation and is suitable for routine application.

Extraction of the enzymatically released DPHP metabolites with *tert*-butyl methyl ether and subsequent derivatisation with HFIP help to achieve a very selective detection of the DPHP metabolites (see Section 11.5 for possible interferences by isomeric DiDP/DiNP metabolites in the case of OH-MPHP). Soft ionisation in negative CI mode allows quantitation limits into the environmental range of measurement, enabling a reliable detection of DPHP exposure in the concentration ranges of both occupational health and environmental medicine. This method was developed within the cooperative project for the promotion of human biomonitoring between the *Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit* (Germany's Federal Ministry for the Environment, Nature Conservation and Nuclear Safety; BMU) and the *Verband der chemischen Industrie* (German Chemical Industry Association; VCI). It has already been published internationally (Gries et al. 2012).

The metabolites of the 2-propylheptyl-2-propyl-4/5-methylhexyl phthalate isomers formed during DPHP production are also determined, provided that cx-MPHxP, OH-MPHP, and oxo-MPHP are formed as oxidised metabolites from the isomers by cleavage of the 2-propyl-4/5-methylhexyl moiety. The metabolites of bis(2-propyl-4/5-methylhexyl) phthalate, which is formed at 1% during DPHP production, cannot be determined using this method.

The developers of this method also developed an analytical method using LC-MS/MS, parallel to the gas-chromatographic methods described herein, as a further option for the quantification of DPHP metabolites (Gries et al. 2012). The LC-MS/MS technique possessed a higher dynamic range of measurement, although somewhat worse limits of quantitation (0.3–0.5 µg/l) were achieved compared to the GC-MS/MS method (0.1–0.3 µg/l). Furthermore, with the LC-MS/MS method, no differentiation was possible between the metabolites of DPHP and those of DiDP. Since the DiDP metabolites present in the urine samples eluate simultaneously to the DPHP metabolites, only a sum parameter can be determined. In contrast, using the GC-HR-MS or GC-MS/MS method, selective measurement of the DPHP metabolites is possible.

Instruments used Agilent GC 5890 gas chromatograph (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) with a CTC A200S autosampler (CTC Analytics AG, Zwingen, Switzerland), and an HR-MS detector (Waters AutoSpec Ultima mass detector, Waters GmbH, Eschborn, Germany); or a GC-MS/MS system (method development: CP-3800/300 MS-TQ, Varian Inc., Palo Alto, USA; external verification: 450 GC/320 MS-TQ, Varian Inc., Palo Alto, USA)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (https://www.dfg.de/en/dfg_profile/statutory_bodies/senate/health_hazards/conflicts_interest/index.html) ensure that the content and conclusions of the publication are strictly science-based.

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